

Diagnostic genetic testing for patients with bilateral optic neuropathy and comparison of clinical features according to *OPA1* mutation status

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Purpose: Inherited optic neuropathy is genetically heterogeneous, and genetic testing has an important role in risk assessment and counseling. The purpose of this study is to determine the prevalence and spectrum of mutations in a group of patients referred for genetic testing to a tertiary center in the United States. In addition, we compared the clinical features of patients with and without mutations in *OPA1*, the gene most commonly involved in dominantly inherited optic atrophy.

Methods: Clinical data and genetic testing results were reviewed for 74 unrelated, consecutive patients referred with a history of insidious, relatively symmetric, bilateral visual loss secondary to an optic neuropathy. Patients were evaluated for disease-causing variants in *OPA1*, *OPA3*, *WFS1*, and the entire mitochondrial genome with DNA sequencing and copy number variation (CNV) testing.

Results: Pathogenic DNA variants were found in 25 cases, with the majority (24 patients) located in *OPA1*. Demographics, clinical history, and clinical features for the group of patients with mutations in *OPA1* were compared to those without disease-causing variants. Compared to the patients without mutations, cases with mutations in *OPA1* were more likely to have a family history of optic nerve disease ($p = 0.027$); however, 30.4% of patients without a family history of disease also had mutations in *OPA1*. *OPA1* mutation carriers had less severe mean deviation and pattern standard deviation on automated visual field testing than patients with optic atrophy without mutations in *OPA1* ($p < 0.005$). Other demographic and ocular features were not statistically significantly different between the two groups, including the fraction of patients with central scotomas (42.9% of *OPA1* mutation positive and 66.0% of *OPA1* mutation negative).

Conclusions: Genetic testing identified disease-causing mutations in 34% of referred cases, with the majority of these in *OPA1*. Patients with mutations in *OPA1* were more likely to have a family history of disease; however, 30.4% of patients without a family history were also found to have an *OPA1* mutation. This observation, as well as similar frequencies of central scotomas in the groups with and without mutations in *OPA1*, underscores the need for genetic testing to establish an *OPA1* genetic diagnosis.

Inherited optic neuropathy is an important cause of blindness worldwide [1]. Genetic optic neuropathies can be inherited as an autosomal dominant (AD), autosomal recessive (AR), X-linked recessive (XR), or mitochondrial (maternal) trait. Dominantly inherited optic atrophy is the most common form with prevalence ranging from 1/10,000 in Denmark to 1/30,000 elsewhere [2-4]. Between 40% and 90% of dominantly inherited optic neuropathy cases are reportedly caused by mutations involving *OPA1* (Gene ID 165500, OMIM 605290) [3,5-9], depending on the population. *OPA1*-related optic neuropathy is typically characterized by insidious symmetric visual loss and dyschromatopsia

beginning within the first two decades of life [2,7]. Optic nerve head pallor and excavation are frequently present, especially in the temporal sector. Syndromic optic neuropathy, which includes neurologic signs and symptoms (such as sensorineural hearing loss and progressive external ophthalmoplegia), has been estimated to occur in 10–30% of patients with disease-causing *OPA1* variants [10,11].

OPA1 is a nuclear gene encoding a dynamin-related GTPase that is ubiquitously expressed and localizes to the inner mitochondrial membrane [12-14]. There are eight alternatively spliced isoforms [15], and disease-causing variants are found throughout the *OPA1* coding sequence. The *OPA1* protein is highly expressed in neuronal tissues, including retinal ganglion cells, and participates in multiple processes, including mitochondrial fission/fusion, oxidative phosphorylation, and apoptosis [16-18]. The molecular and cellular

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mechanisms responsible for *OPA1*-mediated optic neuropathy are only partially understood, and clinical studies of patients with mutations in *OPA1* have been limited with respect to the clinical features examined [3,5-9,19,20]. More than 300 independent disease-causing *OPA1* variants have been characterized (Mitodyn) [21,22]. Although the majority of mutations in *OPA1* are missense or nonsense alleles, approximately 10–13% of patients with inherited optic atrophy have been reported to have large-scale chromosomal deletions or insertions (copy number variants, or CNVs) affecting *OPA1* [23].

Inherited forms of optic neuropathy are also caused by mutations involving mitochondrial DNA-encoded genes *ND4* (OMIM 516006) and *ND6* (OMIM 516006) in patients with Leber hereditary optic neuropathy (LHON; OMIM 535000), mutations in *OPA3* (OMIM 606580) in AD optic atrophy associated with cataract (OMIM 1653000), and mutations in *WFS1* (OMIM 606201) in AR optic atrophy associated with sensorineural hearing loss, diabetes mellitus, and diabetes insipidus (OMIM 222300) [24,25]. Nine other genes are rare causes of optic atrophy inherited primarily as AR traits [4,26-29].

Genetic counseling can be challenging for many optic atrophy families that are too small to confidently differentiate AD, AR, and mitochondrial inheritance from inspection of the pedigree. Genetic testing can help inform genetic counseling for these patients and their families by revealing the disease-causing variant and associated inheritance pattern. Additionally, genetic testing can identify specific mutations that in some cases may be relevant to disease outcomes. Although genetic testing for optic atrophy is offered by several Clinical Laboratory Improvement Amendments (CLIA)-certified laboratories, the mutation detection rates, classifications of disease-causing variants, and clinical features of mutation carriers have not been comprehensively reported [30].

We selected a cohort of consecutively identified patients referred to our genetic testing laboratory who were predominately referred from a single clinical service with a history of insidious, relatively symmetric, bilateral visual loss secondary to an optic neuropathy. We report on the mutation prevalence and variant spectrum for this group of unrelated patients and compare the clinical features of patients with disease-causing *OPA1* variants with cases without an identified genetic cause of disease.

METHODS

Study design and subjects: The Institutional Review Board at the Massachusetts Eye and Ear Infirmary (MEEI) approved this Health Insurance Portability and Accountability Act

(HIPAA)-compliant study that adhered to the Declaration of Helsinki and to the ARVO statement on human subjects. Seventy-four unrelated patients with presumed inherited optic atrophy referred for genetic testing between 2006 and 2014 were included in the study. Most patients were referred from the MEEI Neuro-Ophthalmology service (61 patients) with the remaining patients referred from the National Ophthalmic Disease Genotyping Network (*eyeGENE*; seven patients), MEEI glaucoma service (one patient), and other physicians not at MEEI (five patients). We obtained written informed consent from each patient.

Patients referred for genetic testing had a clinical presentation of insidious, generally symmetric bilateral visual loss in the context of optic nerve pallor and increased cupping that, in the absence of an alternative etiology such as metabolic condition, toxic disorder, neoplastic or paraneoplastic disorder, inflammatory disease (including demyelination), or infection, was suggestive of optic atrophy on a genetic basis. Genomic DNA was extracted from blood, buccal swab, or mouth wash samples according to approved protocols [31]. Blood was obtained by venous puncture (usually from the antecubital vein) into two 10 ml heparinized tubes. Lymphocytes pellets were prepared from each tube and DNA was extracted from each lymphocyte pellet using established protocols [31]. DNA samples were stored at -80 °C until used for sequencing.

Genetic testing: Genetic testing was done at the MEEI CLIA-certified genetic diagnostic laboratory (CLIA number 22D1037777). Initially, the genetic testing procedures for optic atrophy were limited to PCR amplification of the *OPA1* coding sequence (NM_130837.2) followed by Sanger sequencing. In 2012, we developed a next-generation sequencing (NGS) test using selective exon capture for *OPA1*, *OPA3*, *WFS1*, and the entire mitochondrial genome (Genetic Eye Disease test, GEDi test; 31). In total (Sanger sequencing and GEDi NGS testing), 74 unrelated patients underwent genetic analysis. Initially, 62 patients were tested for *OPA1* variants using Sanger sequencing and *OPA1* multiplex ligation-dependent probe amplification (MLPA), and point variants or CNVs were found in 21 patients (Appendix 1). Sufficient DNA was not available for further analysis in three additional cases. The remaining 38 patients (62 – minus 21 cases where mutations were found and minus the three cases without sufficient DNA) were further studied using the NGS GEDi test, along with an additional 12 patients who were newly referred for testing. The additional 12 cases who were taken directly to GEDi testing also had an evaluation for CNVs using *OPA1* MLPA. The GEDi testing identified three additional *OPA1* point mutations and a single case

with a mitochondrial DNA mutation known to cause LHON (m.11778G>A) in the combined group of 38 patients from Sanger and the newly referred 12 patients. If a disease-causing variant was identified, then testing of family members was offered. For patients with *OPA1* variants, five affected family members with variants were identified, and these cases were included in the comparison of clinical features.

For the Sanger sequencing test, all *OPA1* exons (NM_130837.2, isoform 8; ENST00000361510) and 100 base pairs of the flanking intron sequence were amplified using a standard nested PCR approach. Oligonucleotides for amplification and sequencing were selected using Primer3 software (provided by Massachusetts Institute of Technology, Cambridge, MA) and were located at least 40 bp from each exon's splice site. Primer sequences are available on request. PCR was performed in a thermal cycler in a total volume of 25 μ l containing 50 ng genomic DNA; 1.5 mM MgCl₂; 200 μ M each of dATP, dCTP, dGTP, and dTTP; 100 ng forward PCR primer, 100 ng reverse PCR primer; 20 mM Tris-HCl (pH 8.4); 50 mM KCl; and 0.5 U Taq DNA polymerase (Platinum Taq; Invitrogen-Life Technologies, Rockville, MD). Cycling conditions were as follows: an initial denaturing step of 5 min at 94 °C; 35 cycles of denaturation (94 °C for 45 s), annealing (primer-specific annealing temperature for 60 s), elongation (72 °C for 45 s), and a final elongation step of 5 min at 72 °C. Amplified genomic DNA was directly sequenced using sequencing chemistries (BIGDYE Terminators; Applied Biosystems Inc., Foster City, CA) and an automated sequencer (model ABI PRISM 3730xl DNA Analyzer; Applied Biosystems Inc.). Next-generation sequencing was performed using the GEDi diagnostic panel that covers the coding and intronic regions of three optic atrophy-related genes (*OPA1*, *OPA3*, and *WFS1*), as well as the mitochondrial genome as previously described [31].

To assess the likelihood of pathogenicity for missense variants, we used the following criteria: rare (<1%) or novel based on the publically available sequence database from the National Heart, Lung, and Blood Institute Exome Sequencing Project (NHLBI ESP) and the Exome Consortium (ExAC), evolutionarily conserved, and expected to be pathogenic based on the results of two online pathogenicity programs: PolyPhen-2 and SIFT [32,33]. Frameshift and nonsense mutations were assessed for pathogenicity using Mutation-Taster [34]. MLPA was used to identify CNVs using a kit from MRC Holland (P229 OPA1 probemix, Amsterdam, the Netherlands). At least three separate MLPA reactions were conducted for each patient. PCR products were separated on Applied Biosystem's ABI 3130XL capillary sequencers. Analysis was performed using the Coffalyser software (MRC

Holland, Amsterdam, The Netherlands). Mitochondrial DNA variants were assessed according to the algorithm outlined by Wang et al. [35], which accounts for allele frequency (<0.2%), degree of heteroplasmy, and matrilinear inheritance. Heteroplasmy was assessed with analysis of the sequence data.

Clinical assessment according to OPA1 variant status: Demographic, medical, and ophthalmic data were extracted from the medical record masked to *OPA1* mutation status. Onset of symptoms was extracted from the history of present illness. Family history was considered positive if an immediate family member or multiple extended family members were reported to have unexplained vision loss or optic atrophy.

The clinical evaluation of each patient was performed by the MEEI Neuro-Ophthalmology service and included best-corrected Snellen visual acuity; pupillary exam; color discrimination using Ishihara pseudoisochromatic plates; applanation tonometry of intraocular pressure (IOP); dilated stereoscopic funduscopy, including subjective assessment of the cup-to-disc ratio (CDR); assessment of visual fields with static perimetry (Humphrey) using the 24-2 threshold program, kinetic (Goldmann) perimetry, or both. Visual acuity recorded in Snellen equivalents was converted to the LogMAR scale. Absolute asymmetry was calculated by taking the absolute LogMAR difference. Normalized asymmetry was calculated by dividing the absolute asymmetry by the averaged acuity between the two eyes. Patients were considered to have dyschromatopsia if they could read the control plate but could not read all Ishihara plates.

Reliable visual fields were available for 47 patients (37 on the Humphrey visual field [HVF] analyzer and ten on the Goldmann perimeter) and were systematically analyzed for each patient by an ophthalmologist masked to *OPA1* mutation status. The visual field analysis was modified from a previously described method [36]. Only visual fields with reliability indices of $\leq 33\%$ fixation losses, $\leq 20\%$ false positive responses, and $\leq 20\%$ false negative responses were used for analysis. From the extracted visual field global indices (pattern standard deviation [PSD] and mean deviation [MD]), absolute and normalized asymmetries were calculated. The pattern of visual loss was characterized as whether it involved the central field (including the cecocentral and paracentral regions), peripheral visual field (including nasal step, Bjerrum, and temporal wedge zones), or both (Appendix 2). These regions were presumed to be abnormal if there was a cluster of three or more contiguous points with retinal sensitivity depression of 0.5 log unit (−5 dB) relative to age-matched controls on the pattern deviation plot. Goldmann fields were interpreted using the same topographical

descriptors by two investigators (EDG, KB) independently with complete agreement.

Statistical analysis and scoring: Statistical analyses were conducted using IBM SPSS Statistics (Version 22) software. Discrete variables were tested using the chi-square (χ^2) test. Continuous variables were tested using the Student *t* test (unpaired; unequal variance assumed). Bonferroni corrections for multiple comparisons were applied for each measure within each of the three analyses, including demographic/historical data, details of the clinical examination, and results of perimetry where indicated. In all cases, a *p* value of less than 0.05 was considered statistically significant. Post-hoc power calculations were completed using G*Power 3 (Appendix 3). As post-hoc power calculations suggested that the study was best powered to detect differences between *OPAI* mutation positive (M+) and *OPAI* mutation negative (M-) cases in visual field parameters (>90% power), and family history of disease (>69% power), we focused on those features.

RESULTS

Overall mutation detection rate: Seventy-four unrelated patients with optic atrophy were tested for clinically relevant variants in up to three genes and the entire mitochondrial genome using Sanger and next-generation sequencing. We used the following criteria to classify nuclear DNA variants as clinically relevant: 1) rare [$<1\%$] in the general population (Exome Aggregate Consortium); 2) predicted to be pathogenic by PolyPhen2, SIFT, and MutationTaster; 3) evolutionarily conserved [genomic evolutionary rate profile, GERP]; and 4) segregation with disease status (if family members were available). Mitochondrial variants were classified according to the algorithm outlined by Wang et al. [35] that includes frequency of the potentially pathogenic allele ($<0.2\%$), measure of heteroplasmy, and matrilineal inheritance (if possible from the pedigree information). Using these criteria, clinically relevant variants (mutations) were identified in 25 of 74 cases overall. Mutations in *OPAI* were found in 24 cases, and a homoplasmic mitochondrial DNA variant (m.11778G>A) known to cause LHON was found in one case (Figure 1, Table 1, Table 2 and Table 3). Mutations in *OPA3* or *WFS1* were not observed in this cohort. The mutations in *OPAI* included five missense alleles, two nonsense, and three insertions or deletions causing a shift of the reading frame (Table 1). The known recurrent deletion at Val958 (chr3:193,384,959–62,delTTAG [15];) was observed in six unrelated cases.

Four point mutations in *OPAI* were novel: two missense variants (Ser358Asn; Glu499Gly) and two insertion/deletions

resulting in premature stop codons and truncated proteins (c.2333_2334insT, Asn700Lysfs*27; c.2881delG, Val883*). None of these mutations were identified in the EXAC database of 60,706 individuals. SIFT and PolyPhen2 predicted that the novel missense alleles are pathogenic, and the GERP scores (5.6 and 5.8, respectively) indicated that the affected amino acids are evolutionarily conserved (Table 2). The patient with the Ser358Asn mutation had an affected mother and grandmother. A family history was not available for the patient with the Glu499Gly allele. The patient with the c.2333_2334insT mutation reported no family history of neurologic disease. The patient with c.2881delG had a maternal grandfather with a similar history of optic atrophy. Insertion and deletion variants cause premature stop codons and were predicted to be disease causing by MutationTaster (Table 2 [34];).

***OPAI* copy number variations:** Copy number variation (large insertions or deletions) was evaluated using MLPA. CNVs involving *OPAI* were identified in six cases that included five deletions and one insertion (Table 3, Figure 1C,D). The deletions ranged in size from three exons to deletion of the entire gene; three of these deletions have not been previously reported. A novel duplication involving exon 18 was identified in one patient.

Clinical features of *OPAI* mutation positive cases compared to mutation negative cases: The clinical features of 24 patients with mutations in *OPAI*, as well as five family members with confirmed mutations, were compared to 49 patients in whom we did not identify a mutation. The patient with the LHON-related mitochondrial DNA variant was not included in the comparison analysis.

In the combined *OPAI* mutation positive (M+) and *OPAI* mutation negative (M-) group, 85% of the cases were self-identified as Caucasian, and 54% were male. Using available information from clinical records, data were collected for visual acuity, family history of disease, age of diagnosis, reason for referral, alcohol and smoking exposure, extraocular neurologic symptoms, dyschromatopsia, relative afferent pupillary defect (RAPD), IOP, CDR, retinal pathology, and visual field defects (Figure 2, Appendix 4). Power calculations (Appendix 3) indicated approximately 70% power to detect significant comparisons between M+ and M- cases for family history, and greater than 90% power to detect differences in visual field parameters: MD and PSD from Humphrey automated visual fields and the location of visual field defects (peripheral). Below, we focus on these clinical features with adequate power for comparison in the M+ and M- cases.

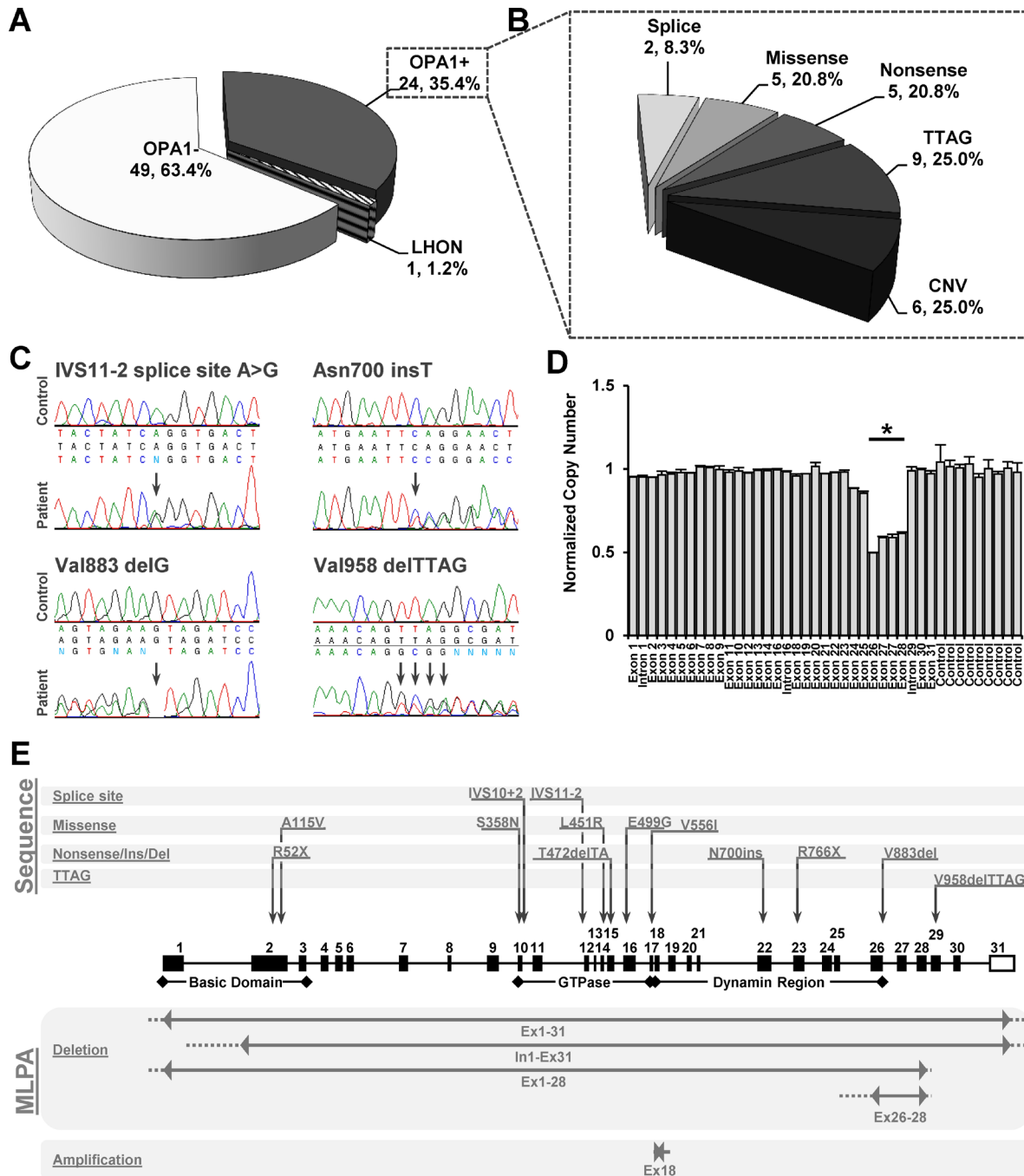


Figure 1. Variants detected in patients with clinically diagnosed optic atrophy. **A:** Pie chart showing the distribution of mutations in the sample by gene and mitochondrial DNA. Numbers represent the number of unrelated patients and the percentage of patients with variants in a gene in the sample. **B:** Distribution of *OPA1* variants by mutation type; percentage among patients with mutations in *OPA1* are included. **C:** Example sequence traces depicting selected variants relative to controls. **D:** Example of a multiplex ligation-dependent probe amplification (MLPA) result depicting a deletion involving exons 26–28. Error bars represent the standard error of the mean; n = 3 MLPA reactions. *depicts p<0.05 compared to control averages. **E:** Schematic of the *OPA1* gene (NM_130837.2). Exons are labeled according to number; the 31st exon is non-coding. Functional domains are indicated immediately below the gene. Sequence variants (above) and copy number variants (below) are indicated according to their position along the *OPA1* gene.

Family history: M+ patients reported earlier age of onset of visual symptoms compared to M- cases (M+: 1.7±1.3 decade; M-: 3.0±2.0 decade, $p = 0.045$; Appendix 4), and 69.6% of M+ patients had a family history of optic atrophy compared to 30.8% of the M- patients ($p = 0.027$). However, of the patients with optic atrophy without a known family history, 30.4% carried an *OPA1* mutation, and 42.9% of patients with a family history of optic atrophy did not have an identified *OPA1* mutation (Appendix 4), arguing that *OPA1* mutation status cannot be inferred based on family history alone.

Visual field defects: Visual field data were available for 93 eyes among 47 patients. The number of unreliable automated HVFs (fixation losses >33%, false positives >20%, or false negatives >20%) was not different between the M+ (13/36 eyes [64%]) and M- groups (16/57 eyes [72%], $p = 0.41$). Mean

deviations (a measure of the overall depression of the visual field) ranged from -0.86 dB to -32.95 dB (Figure 3A). M+ patients had less severe mean deviation compared to the M- patients (M+: -3.88±5.83 dB; M-: -11.49±10.04 dB, $p < 0.005$; Table 4). There was little asymmetry in the mean deviation between the eyes of the M+ and M- patients (Appendix 4; Figure 3B). Pattern standard deviation, a measure of the depth of a localized visual field defect, ranged widely among patients, from 1.48 dB to 12.19 dB (Figure 3C). Average PSDs were statistically significantly lower in the M+ patients compared to the M- patients (M+: 2.29±1.03 dB; M-: 5.49±3.53 dB, $p < 0.005$; Table 4). There was little asymmetry in PSD between the eyes of the M+ and M- patients (Appendix 4; Figure 3D).

TABLE 1. SUMMARY OF *OPA1* POINT MUTATIONS.

N	Mutation type	Nucleotide change	Protein position	ExAC MAF	Reference	Neurologic symptoms
1	Splice	chr3:193,360,552, A>G	IVS11-2 Splice	NP	[40]	None
1	Splice	chr3:193,355,072, T>A	IVS10+2 Splice	NP	Mavrogiannis LA, Clayton-Smith J, Charlton RS (unpublished)*, UK	None
1	Missense	chr3:193,332,823, C>T	Ala115Val	SA 0.00073 ENF 0.00026 Lat 0.00017	[11]	None
1	Missense	chr3:193,361,208, T>G	Leu451Arg	NP	[41]	Neuropathy
1	Missense	chr3:193,363,399, G>A	Val556Ile	NP	[8]	None
1	Missense	chr3:193,355,778, G>A	Ser358Asn	NP	Novel	None
1	Missense	chr3:193,361,782, A>G	Glu499Gly	NP	Novel	Hearing Loss, ophthalmoplegia, myopathy, ataxia
1	Nonsense	chr3:193,332,633, C>T	Arg52X	NP	[42]	Hearing Loss
1	Nonsense	chr3:193,374,986, C>T	Arg766X	NP	[15]	None
1	Insert/ Delete	chr3:193,361,355-6, del TA	Thr472 (564X)	NP	[5]	Paresthesias
1	Insert/ Delete	chr3:193,372,737-8, ins T	Asn700 (727X)	NP	Novel	None
1	Insert/ Delete	chr3:193,380,737, del G	Val883 (883X)	NP	Novel	None
6	delTTAG	chr3:193,384,959-62, del TTAG	Val958 (964X)	Afr 9.96×10 ⁻⁵ ENF 4.62×10 ⁻⁵	[14]	Myopathy, neuropathy, ataxia

*This mutation was included in an online *OPA1* mutation database (MitoDyn) without a publication reference, but acknowledged Mavrogiannis LA, Clayton-Smith J, Charlton RS (unpublished). Abbreviations: N, number with mutation; ExAC, Exome aggregate consortium; MAF, minor allele frequency; NP, Not Present; SA, South Asian; ENF, European Nonfinnish; Lat, Latino; Afr, African; Chr, chromosome; IVS, intervening sequence (intron); X, stop codon; Ala, alanine; Arg, arginine; Glu, Glutamate; Leu, Leucine; Ser, Serine; Thr, Threonine; Val, Valine.

TABLE 2. PATHOGENICITY DATA FOR NOVEL *OPAI* POINT MUTATIONS.

N	Mutation type	Nucleotide change	Mutation	MAF (ExAC)	SIFT/ Polyphen2	Mutation taster	GERP
1	Missense	chr3:193,355,778, G>A	Ser358Asn	NP	0.00/1.00 D/PD	DC	5.6
1	Missense	chr3:193,361,782, A>G	Glu499Gly	NP	0.00/1.00 D/PD	DC	5.8
1	Insert/ Delete	chr3:193,372,737–8, ins T	Asn700 (727X)	NP	NA	DC	NA
1	Insert/ Delete	chr3:193,380,737, del G	Val883 (883X)	NP	NA	DC	NA

Abbreviations: MAF (minor allele frequency); ExAC (Exome aggregate consortium); GERP (Genomic evolutionary rate profile). SIFT results are D Damaging; Polyphen2 results are PD, Probably Damaging; Mutation Taster results are DC, disease causing. Mutation Taster scores are only provided for missense alleles. GERP values range from -12.3 to 6.17, with the positive values representing higher degrees of evolutionary conservation. GERP scores are only applicable to missense alleles. The PolyPhen-2 score is reported as a range from 1.0 to 0.0 with the threshold for “probably damaging” from 1.0 to 0.85, “possibly damaging” from 0.85 to 0.20, and less than 0.20 is designated as “benign.” Sift scores ranges from 0 to 1, and ‘damaging’ are scores less than 0.05, while ‘tolerated’ are scores >0.05. Mutation Taster scores range from 0.0 to 215 and is based on the Grantham Matrix.

Manual Goldmann visual field data were available for 20 eyes among ten patients and were included with the visual field loss pattern analysis along with the HVF data. Although most M+ patients had a localized visual field defect (57%, Table 4), only 42.9% had central, paracentral, or centrocecal scotomas. There was no difference in the frequency of central, paracentral, or centrocecal scotomas between the M+ and M- groups ($p = 0.248$; Table 4). In contrast, the peripheral patterns of field loss (including nasal step, Bjerrum, and temporal wedge patterns) were significantly more common among eyes without mutations in *OPAI* (29/47 [62%]) and were infrequently seen in the M+ patients (5/35 [14.3%]; $p < 0.005$; Table 4).

DISCUSSION

This study reports the clinical profile and genetic testing results for a cohort of patients with insidious, relatively symmetric, bilateral optic neuropathies referred to a tertiary care center in the United States. Genetic testing revealed disease-causing variants in 34% of unrelated cases, with the majority of variants involving *OPAI* (24 of 25). The testing also identified eight novel mutations in *OPAI*, including four point mutations and four CNVs.

Genetic testing in this cohort of patients with optic atrophy provided an opportunity to compare clinical features of patients with mutations in *OPAI* to affected patients without mutations in known disease-causing genes. A family

TABLE 3. SUMMARY OF *OPAI* COPY NUMBER VARIANTS (CNVs).

N	OPAI CNV	CNV size and genomic location		Reference	Neurologic symptoms
		Minimum	Maximum		
2	Deletion, Whole Gene	Unable to determine, all <i>OPAI</i> probes deleted	Unable to determine, all <i>OPAI</i> probes deleted	[38]	None
1	Deletion, Intron 1-Exon 31	Chr3:193,331,962–193,412,551	Chr3:193,311,286-NA*	Novel; Similar in [38]	Transient Hemiplegia
1	Deletion, Exons 1–28	Chr3:193,311,286–193,384,111	NA**-Chr3:193,384,911	Novel; Similar in [38]	None
1	Deletion, Exons 26–28	Chr3:193,380,681–193,384,111	Chr3:193,377,299–193,384,911	Novel; Similar in [23]	None
1	Insertion, Exon 18	Chr3:193,363,781–193,363,804	Chr3:193,362,732–193,364,928	Novel	Hearing Loss

Abbreviations: N, number of mutations; Chr, chromosome. *Cannot determine the 3' end of the deletion because the last *OPAI* probe included in the MLPA set is deleted. **Cannot determine the 5' end of the deletion because the first *OPAI* probe included in the MLPA set is deleted.

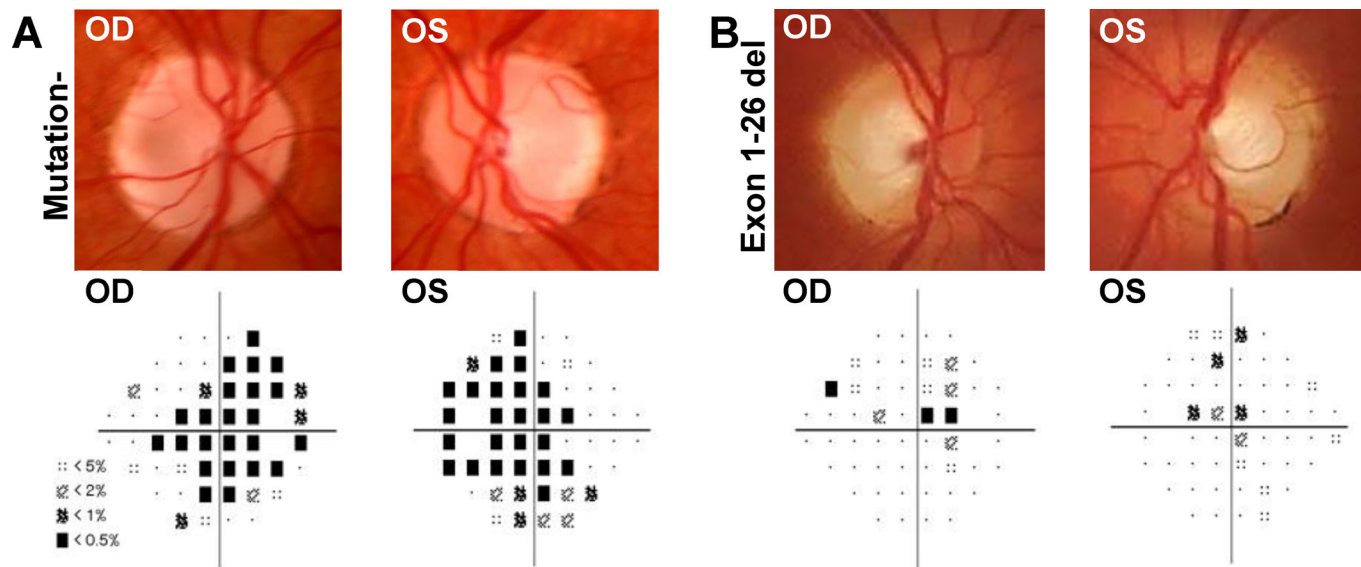


Figure 2. Optic disc and Humphrey visual field deviations in patients with and without mutations in *OPA1*. **A, B**: Selected examples of fundus photographs of optic discs demonstrating temporal pallor (top) and corresponding Humphrey visual field (HVF) pattern standard plots (bottom) in two patients with optic atrophy: one without an *OPA1* variant (or other known gene; **A**), and one with a deletion extending from exon 1 to 28 (**B**). The HVF pattern standard plots show bilateral central/paracentral, temporal, and inferior Bjerrum defects for the mutation negative patient (**A**) and bilateral centrocecal defects for the mutation positive patient (**B**).

history of optic atrophy is more common in *OPA1* mutation carriers; however, 30.4% of *OPA1* mutation positive cases did not report a family history of disease, and 30.8% of *OPA1* mutation negative patients had affected family members. Thus, *OPA1* mutation status cannot be presumed from family history alone. A lack of family history in *OPA1* mutation carriers could be due to the impact of modifier alleles, de novo mutations, or variable disease expressivity due to stochastic effects. Further study of families without history of disease but with at least one member with a mutation in *OPA1* would be interesting and could help differentiate among these possible explanations. Positive family history in *OPA1*-negative patients supports the hypothesis that other, yet undiscovered, genes are likely to cause this clinical phenotype of inheritable disease.

Differences between *OPA1* M+ and M- patients were also observed in visual field data. The overall visual field compromise (i.e., mean deviation score) was greater in patients without mutations in *OPA1* compared with those patients with disease-causing variants. Interestingly, although central scotomas are described as a cardinal feature of *OPA1*-mediated optic atrophy due to the involvement of the retinal ganglion cells in the central maculopapillary bundle [37], only 42.9% of the *OPA1* mutation carriers had visual field defects involving the central visual field. Moreover, a significant difference in the frequency of central scotomas between the *OPA1* mutation positive and negative groups

was not observed. However, patients without mutations in *OPA1* were more likely to have peripheral visual field defects, which suggests that this group of patients, which likely includes multiple etiologies, comprises optic neuropathies that preferentially disrupt retinal ganglion cells outside of the maculopapillary bundle. Other clinical features were not significantly different between the M+ and M- groups, underscoring the value of genetic testing to accurately identify carriers of clinically relevant variants.

Overall, fewer mutations in *OPA1* (32%) were found among patients in this sample compared to other studies (40–90% [3,5-9]). Many of the previously published studies included only European populations with likely *OPA1* founder effects, especially in Denmark [3]. The rate of mutations in *OPA1* in the present study is consistent with one other available study from the United States [9]. Some studies with a higher rate of *OPA1* variants included only patients with a known family history of optic atrophy which likely explains the higher percentage of patients with dominantly inherited mutations in *OPA1*. Importantly, the present study includes all patients referred for genetic testing during the designated time period, regardless of family history, which presumably lowers this type of bias. Among the patients with a positive family history of optic neuropathy, mutations in *OPA1* were found in 57.1% of cases, comparable to most European studies evaluating frequency of mutations in *OPA1* [6,8,9,38]. By comparison, the mutation rate for mitochondrial variants

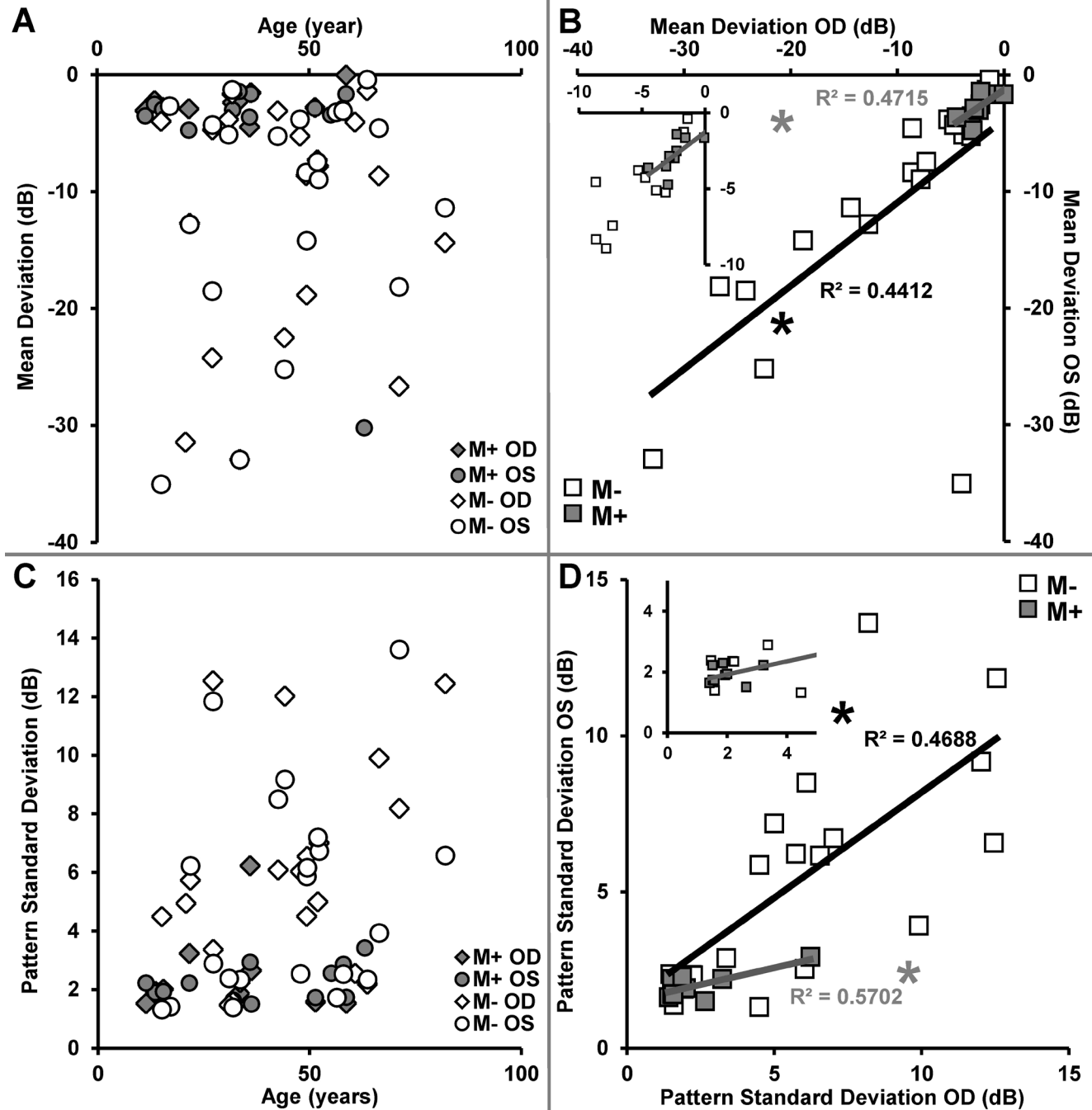


Figure 3. HVF parameters in patients with and without mutations in *OPA1*. **A, B:** Humphrey visual field (HVF) mean deviation values averaged oculus uterque (OU) as a function of age (**A**) and plotted oculus dexter (OD) and oculus sinister (OS) on the X- and Y-axes, respectively (**B**). **C, D:** HVF pattern standard deviation values averaged OU as a function of age (**C**) and plotted OD and OS on the X- and Y-axes, respectively (**D**).

causing LHON was lower than expected [6], although some cases with a history of sequential disease were prescreened for mitochondrial variants by other methods, which could have reduced our opportunity to observe the LHON disease-causing variants in this cohort.

Eight of the variants identified in this study are novel, including novel CNVs. Several other studies have suggested that between 10% and 19% of the mutations in *OPA1* are CNVs [3,4,23,38,39]. The present study supports the observation that *OPA1* CNVs are relatively common, and collectively,

TABLE 4. HUMPHREY VISUAL FIELD (HVF) RESULTS BY *OPAI* MUTATION STATUS.

Visual field parameter	All (N)	<i>OPAI</i> mutation positive (M-),	<i>OPAI</i> mutation negative (M+)	<i>p</i> value (test)
HVF Mean Deviation (dB)	-8.76±9.45 (64)	-11.49±10.04 (41)	-3.88±5.83 (23)	<0.005 (<i>t</i> test)
HVF Pattern Standard Deviation (dB)	4.34±3.27 (64)	5.49±3.53 (41)	2.29±1.03 (23)	<0.005 (<i>t</i> test)
Central/Centrocecal Scotoma (%)	56.1% (46/82)	66.0% (31/47)	42.9% (15/35)	0.185 (χ^2)
Peripheral Field Loss Patterns (%)	40.2% (33/82)	61.7% (29/47)	14.3% (5/35)	<0.005 (χ^2)

N reflects the number of eyes tested. HVF (Humphrey visual fields) inclusion depended on the following reliability parameter limits: Fixation losses (33%), False positives (20%), False negatives (20%). Field loss patterns were assessed for each eye individually and include GVF (Goldmann visual field) interpretations in addition to HVFs. Some Mutation- patients had both central and peripheral defects, and some Mutation+ patients did not have detectable defects on visual field testing. Standard deviations are provided for quantified parameters. Bonferroni-adjusted *p* values (5 measures). Abbreviations: M-, *OPAI* mutation negative, M+, *OPAI* mutation positive.

these results indicate that analysis for *OPAI* CNVs is a necessary component of *OPAI* genetic testing platforms [9].

Determining if a novel DNA variant is the likely cause of disease is a critical aspect of genetic testing. Classification of pathogenic variants depends on allele frequencies in general populations, appropriate control data, evaluating familial segregation data (if available), and functional information. In this study, we used the most rigorous approach possible to classify the newly discovered variants as pathogenic. This approach utilizes the ExAC database of more than 60,000 European Caucasians to assess the allele frequencies of the novel point mutations. All of the novel variants were absent from the ExAC database suggesting that they are exceedingly rare in the general population, as would be expected for a disease-causing variant. Additionally, we relied on evidence of evolutionary conservation and predicted pathogenicity using the well-characterized *in silico* pathogenicity programs SIFT, PolyPhen2, and MutationTaster. All the novel variants are evolutionarily conserved and predicted to be pathogenic. Familial segregation is helpful for establishing pathogenicity if available, but this was infrequently available in our cohort. Future studies to develop standardized high-throughput assays for *OPAI* function would be a valuable tool to assess functional consequences of newly discovered DNA sequence variants.

A strength of this study is the detailed clinical profile for most of cases, making clinical comparisons possible. The present study also has some limitations. First, although the genetic testing approach is comprehensive, it is possible that some existing *OPAI* variants were not detected in the *OPAI* mutation negative group. Potential false-negatives would likely be rare events that would not significantly impact the

overall results. Second, although the sample size was considerable, the study was under-powered to detect differences in many clinical parameters between the two groups. Third, the retrospective design of this study carries its inherent limitations in selection bias and the ability to detect clinical features that might have been relevant in the comparative analysis. Overall, we believe that these limitations do not undermine the general conclusions.

This study reports the prevalence of mutations in currently known genes for a group of patients with insidious, bilateral, relatively symmetric optic neuropathy who were referred for genetic testing because of suspicion of a genetic etiology. The study adds eight novel variants and new detailed clinical information to the current knowledge of *OPAI*-related inherited optic atrophy. Our study also supports the concept that a clinical examination alone is not sufficient to identify *OPAI* variant carriers, and that genetic testing is necessary to establish a genetic diagnosis that informs counseling for these patients and their families. In the future, routine use of genetic testing in patients with this phenotype also might provide a necessary step toward therapeutic opportunities.

APPENDIX 1. SUMMARY OF GENETIC TESTING FOR UNRELATED OPTIC NEUROPATHY PATIENTS.

74 unrelated optic neuropathy patients were enrolled in the study. 62 of these initially underwent *OPAI* genetic testing using Sanger sequencing (NM_130837.2) and *OPAI* MLPA (Multiplex Ligation-dependent Probe Amplification). 38 of the 41 patients without mutations identified by *OPAI* sequencing and MLPA were taken forward for Genetic Eye Disease (GEDi) next-generation sequencing (NGS) testing

(sufficient DNA for GEDi testing was not available for 3 patients). Additionally, 12 patients underwent GEDi testing directly, as well as *OPA1* MLPA. After testing was completed *OPA1* mutations were identified in 24 patients (point mutations and CNVs) and a mitochondrial DNA mutation associated with Leber Hereditary Optic Neuropathy (LHON; m.11778G>A) was identified in one patient. Mutations in *OPA3* and *WFS1* were not identified in this cohort. To access the data, click or select the words “[Appendix 1](#)”.

APPENDIX 2. PARACENTRAL AND PERIPHERAL VISUAL FIELD LOSS DEFINITIONS FOR VISUAL FIELDS SCORING.

Representative plot of each point tested for (A) paracentral visual field loss, (B) cecocentral visual field loss, and (C) peripheral visual field loss in 3 right eyes. (A) The blue boxes indicate the central/paracentral visual field loss regions. A cluster of 3 or more points with sensitivity of -5 dB or more within the central 9 degree of visual field or a single point with P less than 0.5% represents a paracentral visual field loss. (B) Cecocentral scotoma were characterized by a cluster of 3 or more points with sensitivity of -5 dB or more including at least the visual field points indicated by numbers 33 and 41 together or 34 and 42 together. (C) The colored boxes indicate each possible peripheral visual field loss region, including nasal step (orange), temporal wedge (green) and arcuate patterns (red). A cluster of 3 or more points with sensitivity of 5 dB or more in any of these regions represents a peripheral visual field loss pattern. There is overlap between the paracentral zone and arcuate areas, consisting of the second row of points. For the arcuate area to be considered involved, there must be at least 1 point in the third row from the top or bottom that has a retinal sensitivity of 5 dB or more. To access the data, click or select the words “[Appendix 2](#)”.

APPENDIX 3. POST-HOC POWER ANALYSIS RESULTS FOR SOCIODEMOGRAPHIC, CLINICAL AND VISUAL FIELD DATA.

Bolded variables were found to be statistically significant (see respective tables for p values). To access the data, click or select the words “[Appendix 3](#)”.

APPENDIX 4. DEMOGRAPHIC, HISTORICAL, AND CLINICAL DATA COLLECTED FOR OPTIC ATROPHY CASES

Standard deviations are provided for quantified parameters. Abbreviations: N, number; OD, right eye; OS, left eye, OU, both eyes; RAPD, Relative Afferent Pupillary Defect ; IOP,

Intraocular Pressure. To access the data, click or select the words “[Appendix 4](#)”.

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